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(54) Title: IDENTIFICATION OF GENE SEQUENCES AND PROTEINS INVOLVED IN VACCINIA VIRUS DOMINANT T CELL EPITOPES

(57) Abstract: The present invention relates to the identification of gene sequences and proteins involved in vaccinia virus dominant T cell epitopes. Two vaccinia virus CD8+ T cell epitopes restricted by the most common human MHC class I allele, HLA-A0201 have been identified. Both epitopes are highly conserved in vaccinia and variola viruses. The induction of the T cell responses following primary vaccination is demonstrated by the kinetics of epitope specific CD8+ T cells in 3 HLA-A0201 individuals. This information will be useful for the design and analyses of the immunogenicity of experimental vaccinia vaccines, and for basic studies of human T cell memory.

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IDENTIFICATION OF GENE SEQUENCES AND PROTEINS INVOLVED IN VACCINIA VIRUS DOMINANT T CELL EPITOPES

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/442,846, filed January 24, 2003. The entire teachings of the above application is incorporated herein by reference.

GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by a grant PO1 AI-49320 and a subcontract, AI-46725 from the National Institutes of Health/National Institute of Allergy and Infectious Diseases. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Immunization with vaccinia virus resulted in long-lasting protection against smallpox and was the successful approach used to eliminate natural smallpox infections worldwide. This accomplishment was achieved without a detailed understanding of human T cell responses to poxviruses. Due to the concern about the potential use of smallpox virus as a bioweapon, smallpox vaccination is currently being reintroduced. However, severe and life threatening complications from vaccination were associated with congenital or acquired T cell deficiencies, but not with congenital agammaglobulinemia. Considering the high incidence of side effects from current smallpox vaccine, the development of a safer, but equally effective vaccine is very important. Thus, it is important to have a detailed understanding of human T cell responses to poxviruses.

Vaccinia-specific $CD4^+$ and $CD8^+$ T cells have been detected in humans and the number of vaccinia virus-specific T cell responses to smallpox vaccine have

been measured. Additionally, an intracellular cytokine staining assay was applied to quantitate and characterize vaccinia-specific T cells in mice. However, no T cell epitopes have been identified in humans or mice systems. One major obstacle is the size of the virus. Vaccinia is a large virus with an approximately 200-kbp DNA genome that has approximately 200 open reading frames.

In order to analyze T cell responses to licensed and experimental smallpox vaccines at the single cell level, it is essential to identify $CD8^+$ T cell epitopes. In addition to emphasizing the importance of T cells in the immunity to smallpox, there is a critical need to develop new vaccines safe for use in T cell deficient populations. This information will be useful for the design and analyses of the immunogenicity of experimental vaccinia vaccines, and for basic studies of human T cell memory.

SUMMARY OF THE INVENTION

The present invention relates to the identification of gene sequences and proteins involved in vaccinia virus dominant T cell epitopes. In one embodiment, the invention provides a method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 74A. In this embodiment, the polypeptide can be selected from the group consisting of MVA189R, Copenhagen B22R, Copenhagen C16L, Bangladesh-1975 D2L, India-1967 D1L, Garcia-1966 B1L, Brighton Red V212 or Zaire-96-I-16 N1R or other homologues of vaccinia and variola virus. In another embodiment, the method can further comprise a second polypeptide comprising peptide 165. In a further embodiment, the immune response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

In another embodiment, the present invention relates to a method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 165. In this embodiment, the polypeptide can be selected from the group consisting of MVA018L, Copenhagen C7L, Tian Tan TC7L, Bangladesh-1975

D11L, India-1967 D8L, Garcia-1966 B14L, Brighton Red V028 or Zaire-96-I-16 D10L or other homologues of vaccinia and variola virus. In a further embodiment, the method can further comprise a second polypeptide comprising peptide 74A. In a further embodiment, the immune response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

In another embodiment, the present invention relates to a method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 74A, immunogenic fragments or mutants thereof. From 1 to about 4 amino acids can be substituted to make up the immunogenic fragments or mutants of peptide 74A, without essentially detracting from the immunological properties of peptide 74A. In this embodiment, the polypeptide can be selected from the group consisting of MVA189R, Copenhagen B22R, Copenhagen C16L, Bangladesh-1975 D2L, India-1967 D1L, Garcia-1966 B1L, Brighton Red V212 or Zaire-96-I-16 N1R or other homologues of vaccinia and variola virus. In another embodiment, the method can further comprise a second polypeptide comprising peptide 165, immunogenic fragments or mutants thereof. In a further embodiment, the immune response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

In another embodiment, the present invention relates to a method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 165, immunogenic fragments or mutants thereof. From 1 to about 4 amino acids can be substituted to make up the immunogenic fragments or mutants of peptide 165, without essentially detracting from the immunological properties of peptide 165. In this embodiment, the polypeptide can be selected from the group consisting of MVA018L, Copenhagen C7L, Tian Tan TC7L, Bangladesh-1975 D11L, India-1967 D8L, Garcia-1966 B14L, Brighton Red V028 or Zaire-96-I-16

D10L or other homologues of vaccinia and variola virus. In another embodiment, the method can further comprise a second polypeptide comprising peptide 74A, immunogenic fragments or mutants thereof. In a further embodiment, the immune response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

The present invention also relates to a method of identifying the presence of vaccinia, variola or other related poxvirus in a sample comprising determining whether T cells present in the sample (*e.g.*, blood, lymph and tissue) become activated in the presence of a polypeptide selected from the group consisting of: peptide 74A (SEQ ID NO: 1), peptide 165 (SEQ ID N O: 2) and a combination thereof, wherein if the T cells become activated, then vaccinia, variola or other related poxvirus is present in the sample. Whether the T cells present in the sample become activated can be determined using, for example, a cytokine assay (*e.g.*, ELISPOT), a flow cytometry assay (*e.g.*, tetramer staining assay)and/or a limiting dilution assay.

T cells can be present in the original sample or can be added to the sample. For example, the sample can be blood which contains T cells. In this embodiment, whether the T cells become activated in the presence of the polypeptide is determined, wherein if the T cells become activated, then vaccinia, variola or other related poxvirus is present in the sample. In another embodiment, the sample does not initially contain T cells. In this embodiment, the sample is contacted with T cells that become activated in the presence of a vaccinia, variola or other related poxvirus. Then whether the T cells become activated in the presence of the polypeptide is determined, wherein if the T cells become activated, then vaccinia, variola and/or other related poxvirus is present in the sample.

The present invention also relates to a method of determining whether an individual has been infected with vaccinia, variola or other related poxvirus comprising determining whether the individual's T cells become activated in the presence of polypeptide selected from the group consisting of: peptide 74A (SEQ ID NO: 1), peptide 165 (SEQ ID N O: 2) and a combination thereof, and wherein if the

individual's T cells become activated in the presence of the peptide, then the individual has been infected with vaccinia, variola or other related poxvirus.

A method of monitoring the effectiveness of a vaccinia, variola or other related poxvirus vaccine in an individual who has been administered the vaccinia vaccine is also encompassed by the present invention. The method comprises determining whether the individual's T cells become activated in the presence of a polypeptide selected from the group consisting of: peptide 74A (SEQ ID NO: 1), peptide 165 (SEQ ID N O: 2) and a combination thereof, wherein if the individual's T cells become activated, then the vaccine is effective in the individual. In one embodiment, the vaccine is a vaccinia virus vaccine. In another embodiment, the vaccinia virus vaccine is a cancer vaccine.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

Fig. 1 is a schematic depicting the kinetics of the frequency of IFN-γ-producing cells specific to these peptides and to whole vaccinia virus quantitated by IFN-γ ELISPOT assay using the peripheral blood mononuclear cells (PBMCs) of the vaccinia-immune donors. Closed circle: peptide 74A-specific cells. Open circle: peptide 165-specific cells. Closed square: vaccinia virus-specific cells. P: pre-immunization.

Fig. 2 depicts FACS staining to confirm specificity of MVA74A and MVA165 tetramers. HLA-A0201-negative donor PBMC was mixed with $CD8^+$ T cell clones specific for either the MVA74A or the MVA165 vaccinia virus epitope at a ratio of 10:1. Four color FACS analysis was done to determine the specificity. Cells are gated on $CD3^+$ $CD4^-$ cells with tetramer on the X-axis and CD8 on the y-axis.

Fig. 3 is a schematic depicting the quantitation of vaccinia virus epitope-specific $CD8^+$ T cells by HLA-A0201/peptide 74A tetramer staining (top) and HLA-A0201/peptide 165 tetramer staining (bottom) of PBMCs of donor 1. Cells were gated on $CD3^+$ and $CD4^-$ cells with tetramer staining on the x-axis and CD8 on the y-axis. The larger squares show $CD8^+$ cells and the smaller squares show $CD8^+$ and $tetramer^+$ cells.

Fig. 4 is a schematic depicting the kinetics of the frequency of $CD8^+$ T cells specific for each epitope quantitated by tetramer staining in PBMCs of three donors after primary immunization. Closed circle: 74A-specific. Open circle: 165-specific. P: pre-immunization. Frequency is calculated per million PBMC for comparison with the data from epitope-specific IFN-γ ELISPOT assays.

DETAILED DESCRIPTION OF THE INVENTION

A description of preferred embodiments of the invention follows.

Successful vaccines deliver to a host one or more antigens derived from a pathogen, thereby stimulating an immune response which protects against subsequent challenge with the pathogen. Such vaccines can take a variety of forms, including attenuated or killed pathogens, for example, viruses or bacteria; one or more proteins or peptides derived from a pathogen or synthetic or recombinant versions of such proteins or peptides; or one or more nucleic acid molecules encoding one or more proteins or peptides from the pathogen, such as a naked DNA vaccine or a nucleic acid molecule administered in a suitable vector, such as a recombinant virus or bacterium or an immunostimulating complex. Vaccines against cell proliferative diseases, such as cancers, typically utilize proteins or fragments thereof, or nucleic acid molecules encoding proteins or fragments thereof, which are unique to diseased cells or generally more abundant in diseased cells compared to healthy cells.

Cell-mediated immunity is dependent upon lymphocytes known as B cells and T cells. B cells produce antibodies targeted against extracellular antigens. T cells recognize antigen fragments (peptides) which are displayed at the surface of a host cell. Such antigen fragments result from uptake of the antigen by a host cell, or

synthesis of the antigen within the host cell, followed by cleavage of the antigen within the cell. Foreign proteins which are synthesized within the host cell or are taken up by the host cell via specific receptors are fragmented within the cytosol of the cell. One or more of the resulting peptides can become associated with class I major histocompatibility molecules (MHC I), and the resulting complexes are then presented at the surface of the cell. These MHC I/peptide complexes are recognized by specific T cell receptors in certain $CD8^+$ T cells, and the peptides so presented are referred to as CD8 epitopes.

A foreign protein can be taken up by a host cell nonspecifically via endocytosis and then fragmented into peptides in a cellular lysosomal or endosomal compartment. One or more of these peptides can then become associated with a class II major histocompatibility molecule (MHC II) to form a complex which is then presented at the surface of the host cell. These MHC II/peptide complexes are recognized by $CD4^+$ T cells expressing a specific receptor which recognizes the MHC II/peptide complex. These peptides are referred to as CD4 epitopes.

Peripheral T cells in the blood and organs of the immune system (e.g. spleen and lymph nodes) exist in a quiescent or resting state. Upon interaction of T cells with an MHC/epitope complex, the T cells proliferate and differentiate into activated cells having a variety of functions. $CD8^+$ T cells typically become cytotoxic upon activation and destroy antigen-presenting cells via direct contact. Activated $CD4^+$ T cells provide a helper function to B cells, enabling B cells to differentiate into antibody-producing cells. Activated $CD8^+$ T cells and $CD4^+$ T cells release a variety of cytokines (lymphokines or interleukins), which can, for example, control differentiation of many classes of lympholytic precursor cells.

The present invention relates to vaccinia virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) lines that were established by limiting dilution cloning from the peripheral blood mononuclear cells (PBMCs) of HLA-A0201-positive donors who received primary immunization with the licensed smallpox vaccine, Dryvax®. Among the highly polymorphic human MHC class I genes, HLA-A0201 was chosen to identify $CD8^+$ T cell epitopes because of the commonality of this allele among most ethnic groups. HLA-A0201 peptide binding motif searches was performed on

all of the protein sequences of the modified vaccinia virus Ankara (MVA) strain (GenBank accession number U94848), which is being proposed for use as an attenuated smallpox vaccine and as a vector for vaccination against other infectious agents. The computer algorithm "HLA Peptide Binding Predictions" (on the World Wide Web at: bimas.dcrt.nih.gov/molbio/hla_bind/ visited on August 20, 2001 and August 21, 2001) was used to calculate the binding affinity (score) of 9mer peptides to the HLA-A0201 molecule. It was hypothesized that early gene products may be more likely to have $CD8^+$ T cell epitopes, since in both humans and mice all of the known $CD8^+$ T cell epitopes to cytomegalovirus are encoded by immediate-early phase proteins. The early, early and late, and late genes in vaccinia were categorized by nucleotide sequence motifs, such as a late promoter or an early termination motif. For initial screening all peptides with; (1) a binding score of more than a 1000 (70 peptides); or (2) a binding score of 100 to 999 and encoded by a gene expressed early or both early and late (125 peptides) were synthesized. A total of 195 peptides were screened using fifteen vaccinia virus-specific CTL lines. Two T cell epitopes were restricted by HLA-A0201 and cross-reactive to MVA.

One CTL line, VA55 3.13, recognized peptide 74A, CLTEYILWV (SEQ ID NO: 1), in a 21.7K protein encoded by a putative early and late gene, "189R", of the MVA strain with a calculated binding score of 3607. Another CTL line, VA49 3.12, recognized peptide 165, KVDDTFYYV (SEQ ID NO: 2), which is in a host range protein encoded by a putative early and late gene, "018L", with a calculated binding score of 365. Figure 1 demonstrates the high level of specific recognition by these CTL lines of their respective epitope peptides (i.e., peptide 74A or peptide 165) in a dose response CTL experiment. These epitope sequences are highly-conserved in vaccinia and variola viruses (Table 1).

	GenBank accession #	Gene name	74A peptide	Gene name	165 peptide
Vaccinia					
MVA	U94848	189R	CLTEYILWV (SEQ ID NO: 1)	018L	KVDDTFYYV (SEQ ID NO: 2)
Copenhagen	M35027	B22R & C16L[b]	*********	C7L	*********
Tian Tan[a]	AF095689			TC7L	*********
Variola major					
Bangladesh-1975	L22579	D2L	*********	D11L	*********
India-1967	X69198	D1L	*********	D8L	*********
Variola minor					
Garcia-1966	Y16780	B1L	*********	B14L	*********
Cowpox					
Brighton Red	AF482758	V212	*********	V028	*********
Monkeypox					
Zaire-96-I-16	AF380138	N1R	*********	D10L	***Y*L*** (SEQ ID NO: 3)

Table 1. Conservation of epitopes among poxviruses causing infection in human Only strains of which complete genome has been sequenced are listed.

* indicate identical amino acid.

[a]Tian Tan strain does not have 189R orthologue according to the nucleotide sequence.

[b]Both genes are located within the inverted terminal repeats and the gene sequences are identical.

Epitope-specific T cell clones can be generated using methods which are generally known in the art (see, for example, Fathman, *et al.*, in Paul, ed., *Fundamental Immunology*, second edition, Raven Press (1989), Chapter 30, the contents of which are hereby incorporated by reference in their entirety). The isolation of epitope-specific T cell clones is based on T cell biology. Generally, an animal, such as a mouse, is immunized with a preparation of antigens (a bacterial lysate, or a purified protein) or is infected with a virus, such as a wild type virus or a recombinant virus containing heterologous genes encoding one or more proteins from a pathogenic microorganism, such as a virus. The animal is then sacrificed and

the peripheral blood mononuclear cells (PBMC: includes T cells, B cells, monocytes), spleen and lymph nodes are isolated. The isolated cells are then cultured in media containing a defined component of the original antigenic preparation, often a recombinant or purified protein, and the essential T cell growth factor interleukin-2 (IL-2). The only T cells which will proliferate are those which recognize MHC/epitope complex in which the epitope is derived from the antigenic preparation. These cells become activated and proliferate while the unactivated cells begin to die. The cultures are maintained for several weeks, with the media containing antigen and IL-2 being periodically replaced. Eventually, clusters of living and dividing cells (a T cell line) can be observed in some of the cultures.

The proliferating cells are generally not clonal at this point and are of limited use for assaying epitope specific T cell responses. The T cell line is, preferably, cloned through a process referred to as limiting dilution. In this method, PBMC are isolated from, for example, the same strain as the original used to isolate the T cell line. These cells, called antigen presenting cells, will serve as a source of MHC proteins and will present the MHC:peptide complex to the T cell line. The T cell line is diluted to a concentration of about 1 to 5 T cells/mL in a suspension of APCs that contains the antigen of interest and IL-2. This suspension is then transferred into, for example, round or "v"-bottom 96 well microtitre plates, so that each well contains, on average, no more than 1 T cell. The cultures are maintained for several weeks and a clone can grow out of one or more cultures. The cells isolated by limiting dilution are the progeny of a single cell that expresses only one T cell receptor, and the clone is thus epitope-specific.

$CD8^+$ T cells specific to these epitopes were measured at several time points following primary immunization by peptide/HLA-A0201 tetramer staining using the PBMCs of three HLA-A0201-positive donors. Figure 2 shows representative FACS plots of donor 1 PBMC. In Figures 3 and 4, "preimmune" means prior to primary first immunization, and "two weeks" means two weeks after the second immunization for donor 3 who failed to "take" after primary immunization, and twenty days after primary immunization was immunized for the second time. In all three donors the frequency of vaccinia-specific $CD8^+$ T cells peaked two weeks after

primary immunization and then declined, but were still detectable one to three years following primary immunization (Figure 3). Two weeks after vaccination the IFN-γ-producing cells specific to these two epitopes were 14% of total vaccinia virus-specific IFN-γ-producing cells in donor 1, 35% in donor 2, and 6% in donor 3 (Figure 4).

Thus, two $CD8^+$ T cell epitopes restricted by HLA-A0201, the most common MHC class I allele in humans have been identified. These are the first T cell epitopes that have been reported for vaccinia virus. IFN-γ-producing cells specific to these two epitopes represented 6 to 35% of total number of IFN-γ-producing cells specific to vaccinia virus. The frequency of epitope-specific T cells was always higher by peptide/HLA tetramer staining than by IFN-γ-ELISPOT assay, although post-vaccination kinetics for each epitope-specific T cell was similar using both methods.

As for epitope selection, peptide 74A was the 15^{th} highest binding peptide to HLA-A0201 of the 195 peptides selected for screening and peptide 165 was the 95^{th} highest binder. One common characteristic of these two peptides is that they are both encoded by genes with a late promoter and an early termination motif, which means they may be expressed at both early and late phases of infection. The 189R gene of MVA strain encoding peptide 74A is a nonessential gene with unknown function. The 018L gene of MVA encoding peptide 165 is an orthologue of the host range protein, C7L, of the Copenhagen strain. Although selection of peptides was biased toward genes expressed in the early phase of infection, viral proteins produced in the early phase of infection may be processed and presented more efficiently by infected cells than those produced only in late phase, as a result of vaccinia virus down regulating host protein synthesis. These two epitopes are highly-conserved among variola viruses, suggesting the CTLs recognizing these epitopes will recognize variola virus-infected cells.

In one embodiment, the invention provides a method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 74A. In this embodiment, the polypeptide can be selected from the group consisting of

MVA189R, Copenhagen B22R, Copenhagen C16L, Bangladesh-1975 D2L, India-1967 D1L, Garcia-1966 B1L, Brighton Red V212 or Zaire-96-I-16 N1R or other homologues of vaccinia and variola virus. In another embodiment, the method can further comprise a second polypeptide comprising peptide 165. In a further embodiment, the immune response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

In another embodiment, the present invention relates to a method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 165. In this embodiment, the polypeptide can be selected from the group consisting of MVA018L, Copenhagen C7L, Tian Tan TC7L, Bangladesh-1975 D11L, India-1967 D8L, Garcia-1966 B14L, Brighton Red V028 or Zaire-96-I-16 D10L or other homologues of vaccinia and variola virus. In a further embodiment, the method can further comprise a second polypeptide comprising peptide 74A. In a further embodiment, the immune response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

In another embodiment, the present invention relates to a method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 74A, immunogenic fragments or mutants thereof. From 1 to about 4 amino acids can be substituted to make up the immunogenic fragments or mutants of peptide 74A, without essentially detracting from the immunological properties of peptide 74A. In this embodiment, the polypeptide can be selected from the group consisting of MVA189R, Copenhagen B22R, Copenhagen C16L, Bangladesh-1975 D2L, India-1967 D1L, Garcia-1966 B1L, Brighton Red V212 or Zaire-96-I-16 N1R or other homologues of vaccinia and variola virus. In another embodiment, the method can further comprise a second polypeptide comprising peptide 165, immunogenic fragments or mutants thereof. In a further embodiment, the immune

response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

In another embodiment, the present invention relates to a method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 165, immunogenic fragments or mutants thereof. From 1 to about 4 amino acids can be substituted to make up the immunogenic fragments or mutants of peptide 165, without essentially detracting from the immunological properties of peptide 165. In this embodiment, the polypeptide can be selected from the group consisting of MVA018L, Copenhagen C7L, Tian Tan TC7L, Bangladesh-1975 D11L, India-1967 D8L, Garcia-1966 B14L, Brighton Red V028 or Zaire-96-I-16 D10L or other homologues of vaccinia and variola virus. In another embodiment, the method can further comprise a second polypeptide comprising peptide 74A, immunogenic fragments or mutants thereof. In a further embodiment, the immune response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

Several methods are described in the literature which are useful for the identification of T cell epitopes. For example, DeLisi *et al.* have suggested that potential epitopic sites may be located by identification of potential amphipathic alpha helical regions in the molecule. DeLisi *et al.*, *Proc. Natl. Acad. Sci. USA 82*:7048 (1987). Bixler *et al.* describe a strategy of synthesizing overlapping synthetic peptides encompassing an entire protein molecule for delineation of T cell epitopes. Bixler *et al.*, *Immunol. Com. 12*:593 (1983); Bixler *et al. J. Immunogenet. 11*:339 (1994). A synthetic method described by Gysen (*Ciba Foundation Symposium 119*:130 (1986)) permits synthesis of a large variety of peptides thereby mimicking of a variety of potential binding sites, in turn allowing rapid scanning of a molecule.

More traditional methods, such as enzymatic or chemical digestion of proteins provide peptide fragments which may be tested for T cell activity. For

example, enzymes such as chymotrypsin, elastase, ficin, papain, pepsin, or trypsin provide limited and predictable fragments by cleavage of specified amino acid linkages; similarly chemical compounds such as N-chloro-succinimide BPNS-skatole, cyanogen bromide, formic acid, or hydroxylamine, also produce definable fragments by their action on proteins. The presence of the desired T cell stimulating activity in any given fragment can be determined by subjecting purified fragments to a standard T cell proliferation assay, or by analyzing unpurified fragments with a T cell Western Assay. Young *et al.*, *Immunol. 59*:167 (1986).

Peptide 74A and peptide 165 of the invention are CD8 epitopes and the T cells specific for these peptides are $CD8^+$ T cells. The effector functions of $CD8^+$ T cells include lysis of antigen presenting cells and release of cytokines. Therefore, the extent of $CD8^+$ T cell response to the antigen presenting cells can be determined using an assay for cell lysis or by measuring the production of one or more cytokines. The $CD8^+$ T cell response can also be measured by measuring the extent of release of one or more cytokines. In general, greater cell lysis activity or cytokine release will correlate with greater immunogenicity.

In one embodiment, the present invention relates to methods for immunizing an individual, particularly a human, against infection by vaccinia and/or variola virus by inducing an immune response against a polypeptide comprising peptide 74A and/or peptide 165. In a further embodiment, the immune response can be induced against a polypeptide comprising an immunogenic fragment or mutant of peptide 74A and/or peptide 165. Although the methods described herein are particularly useful for human immunization, the methods are equally applicable to other mammals. In particular embodiments, the individual is positive for the HLA-A0201 gene.

As used herein the terms "immunogenic fragment" and "mutant" of peptide 74A and/or peptide 165 refer to polypeptides in which 1 to about 4 amino acids have been substituted without essentially detracting from the immunological properties thereof can be generated in a variety of ways. For example, *in vitro* mutagenic techniques can be used to modify the cloned gene encoding peptide 74A and/or peptide 165. Such methods, which are well known to one skilled in the art, can be

used to delete, insert or substitute nucleotides in the gene resulting in the deletion, insertion or substitution of amino acids in the encoded product. Examples of immunogenic fragments or mutants of peptide 74A and peptide 165 include, but are not limited to, those shown in Table 2. The immunological properties of the mutagenized encoded product can be assayed using methods such as those which are well known to one skilled in the art.

Possible Immunogenic fragments or mutants	74A peptide	165 peptide
1	ILTEYILWV (SEQ ID NO: 4)	IVDDTFYYV (SEQ ID NO: 19)
2	LLTEYILWV (SEQ ID NO: 5)	LVDDTFYYV (SEQ ID NO: 20)
3	FLTEYILWV (SEQ ID NO: 6)	FVDDTFYYV (SEQ ID NO: 21)
4	CLAEYILWV (SEQ ID NO: 7)	KVADTFYYV (SEQ ID NO: 22)
5	CLYEYILWV (SEQ ID NO: 8)	KVYDTFYYV (SEQ ID NO: 23)
6	CLFEYILWV (SEQ ID NO: 9)	KVFDTFYYV (SEQ ID NO: 24)
7	CLTEIILWV (SEQ ID NO: 10)	KVDDIFYYV (SEQ ID NO: 25)
8	CLTEKILWV (SEQ ID NO: 11)	KVDDKFYYV (SEQ ID NO: 26)
9	CLTENILWV (SEQ ID NO: 12)	KVDDYFYYV (SEQ ID NO: 27)
10	CLTEYIAWV (SEQ ID NO: 13)	KVDDTFAYV (SEQ ID NO: 28)
11	CLTEYIYWV (SEQ ID NO: 14)	KVDDTFHYV (SEQ ID NO: 29)
12	CLTEYIHWV (SEQ ID NO: 15)	IVADTFYYV (SEQ ID NO: 30)
13	ILAEYILWV (SEQ ID NO: 16)	IVADIFYYV (SEQ ID NO: 31)
14	ILAEIILWV (SEQ ID NO: 17)	IVADIFAYV (SEQ ID NO: 32)
15	ILAEIIAWV (SEQ ID NO: 18)	LVYDKFHYV (SEQ ID NO: 33)

Table 2. Examples of immunogenic fragments and mutants of peptide 74A and peptide 165.

Effective dosages for inducing an immune response (also referred to as a virus protective response) against vaccinia and/or variola can be determined empirically with initial dosage ranges based upon historical data for peptide/protein vaccine compositions. As used herein, the terms "induced immune response" or "virus protective response" refers to an immunological response in the individual resulting in the successful control or limitation of infection by vaccinia and/or variola virus which is clinically observed.

For example, individuals can be administered dosages of peptide 74A and/or peptide 165 ranging from 0.5-500 micrograms. Whether a particular dosage is effective can be determined using well known T cell proliferation and cytotoxicity assays. For example, following administration of the protein to an individual blood is drawn. Cytotoxic T cells are identifiable by ^{51}Cr release assay (see e.g., Kuwano *et al.*, *J. Virol. 140*:1264-1268 (1988)). Helper T cells are identifiable by a standard T cell proliferation assay (see e.g., Kurane *et al.*, *J. Clin. Invest. 83*:506-513 (1989)). The results from these studies are compared with results from the same experiments conducted with T cells from the same individual prior to administration of the antigen. By comparing this data, effective dosage ranges can be determined.

A wide variety of pharmaceutically acceptable carriers are useful. Pharmaceutically acceptable carriers include, for example, water, physiological saline, ethanol polyols (e.g., glycerol or administration is typically parenteral (i.e., intravenous, intramuscular, intraperitoneal or subcutaneous). An adjuvant (e.g., alum) can also be included in the vaccine mixture.

The invention also pertains to a method for immunizing an individual against infection by vaccinia and/or variola virus by administering a vaccine composition comprising at least one essentially pure T cell epitope (i.e., peptide 74A or peptide 165) in combination with a pharmaceutically acceptable carrier. Due to genetic variability between individuals, a single T cell epitope may not stimulate a virus protective response in all individuals to whom it is administered. Therefore, by combining two or more distinct T cell epitopes (i.e., both peptide 74A and peptide 165), the vaccine is more broadly effective. As indicated above, helper T cell epitopes and cytotoxic T cell epitopes are thought to comprise distinct (albeit possibly overlapping) regions of proteins. Cytotoxic T cell epitopes can be distinguished from helper T cells epitopes experimentally using the cytoxicity and proliferation assays described above (helper T cells stimulate proliferation but do not posses cytotoxic activity).

Peptide 74A and/or peptide 165 can be administered as an polypeptide. Such polypeptides can be synthesized chemically. Alternatively, a truncated portion of a gene encoding peptide 74A and/or peptide 165 can be expressed in a cell, and the

encoded product can be isolated using know methods (e.g., column chromatography, gel electrophoresis, etc.).

As used herein, the term polypeptide means any amino acid sequence which is identical or substantially homologous to peptide 74A and/or peptide 165. The expression substantially homologous refers to polypeptides having an amino acid sequence of peptide 74A or peptide 165 in which amino acids have been substituted without essentially detracting from the immunological properties thereof. This definition includes amino acid sequences of sufficient length to be classified as oligopeptides (these terms are not used consistently or with great precision in the literature).

In one embodiment, both a helper T cell epitope and a cytotoxic T cell epitope can be administered to the individual. The stimulation of cytotoxic T cells is desirable in that these cells will kill cells infected by vaccinia and/or variola virus. The stimulation of helper T cells is beneficial in that they secrete soluble factors which have a stimulatory effect on other T cells, as well as B cells.

In another embodiment, a gene encoding a protein listed in Table 1, or a portion thereof which contains peptide 74A or peptide 165, can be cloned into a recombinant virus which expresses peptide 74A or peptide 165, or immunogenic fragment or mutant thereof, in the individual to be immunized. An example of such a recombinant virus system is the vaccinia system described by Paoletti *et al.* (U.S. Patent No. 4,603,112), the disclosure of which is incorporated herein by reference. Other viruses have been described in the literature which have a genome which can accommodate the insertion of a foreign DNA such that a protein encoded by the DNA is expressed *in vivo*. Any such recombinant virus is useful for the practice of this invention.

Identification of these epitopes will enable the analysis and quantitation of vaccinia virus-specific $CD8^+$ T cells in the acute and memory phases and to compare $CD8^+$ T cell responses specific to different epitopes. Additionally, expansion and subsequent shrinkage of epitope-specific $CD8^+$ T cells at the T cell receptor level can be monitored. Definition of T cell epitopes will help us to better understand human T cell responses to vaccinia virus as a model of human infection. In addition, it will

provide a quantitative measure of poxvirus T cell immunity when these viruses are used as viral vectors.

The present invention also relates to a method of identifying the presence of vaccinia, variola virus and/or other related poxvirus in a sample comprising determining whether T cells present in the sample become activated in the presence of a polypeptide selected from the group consisting of: peptide 74A (SEQ ID NO: 1), peptide 165 (SEQ ID N O: 2), an immunogenic mutant and fragment thereof and a combination thereof, wherein if the T cells become activated, then vaccinia, variola and/or other related poxvirus is present in the sample. In a particular embodiment, the T cells are CD8+ T cells.

As used herein a "sample" for use in the methods of the present invention can be any type of sample that can be analyzed in the method and can be obtained from a variety of sources. T cells can be present in the original sample or can be added to the sample. The sample can be one which is found in any environment, such as an unknown powder or liquid. In addition, the sample can be obtained from a host, such as a mammalian host or individual (*e.g.*, human, canine, feline, bovine, murine). Samples from a host include blood (*e.g.*, whole blood, PMBCs), lymph (*e.g.*, lymph fluid) and tissue (*e.g.*, lymph nodes, spleen). In a particular embodiment, the sample is from an individual that is positive for the HLA-A0201 gene.

For example, the sample can be a sample which does not initially contain T cells. In this embodiment, the sample is contacted with T cells that become activated in the presence of a vaccinia, variola and/or other related poxvirus. Then whether the T cells become activated in the presence of the polypeptide is determined, wherein if the T cells become activated, then vaccinia, variola and/or other related poxvirus is present in the sample. In another embodiment, the sample can be blood which contains T cells. In this embodiment, whether the T cells become activated in the presence of the polypeptide is determined, wherein if the T cells become activated, then vaccinia, variola and/or other related poxvirus is present in the sample.

Thus, the present invention also relates to a method of determining whether an individual has been infected with vaccinia, variola virus and/or other related pox virus comprising determining whether the individual's T cells become activated in the presence of polypeptide selected from the group consisting of: peptide 74A (SEQ ID NO: 1), peptide 165 (SEQ ID N O: 2), an immunogenic mutant or fragment thereof and a combination thereof, and wherein if the individual's T cells become activated in the presence of the peptide, then the individual has been infected with vaccinia, variola and/or other related poxvirus.

As described herein, peripheral T cells in the blood and organs of the immune system (e.g. spleen and lymph nodes) exist in a quiescent or resting state. Upon interaction of T cells with an MHC/epitope complex, the T cells proliferate and differentiate into activated cells having a variety of functions. $CD8^+$ T cells typically become cytotoxic upon activation and destroy antigen-presenting cells via direct contact. Activated $CD4^+$ T cells provide a helper function to B cells, enabling B cells to differentiate into antibody-producing cells. Activated $CD8^+$ T cells and $CD4^+$ T cells release a variety of cytokines (lymphokines or interleukins), which can, for example, control differentiation of many classes of lymphoblytic precursor cells.

Whether the T cells present in the sample become activated can be determined using a variety of assays known to those of skill in the art. For example, a cytokine assay (*e.g.*, ELISPOT), a flow cytometry assay (*e.g.*, tetramer staining assay), intracellular cytokine staining assay (ICS) and/or a limiting dilution assay (LDA) can be used in the methods of the present invention.

Poxviruses such as vaccinia virus allow for simplified integration of multiple foreign genes with high levels of expression, and thus, are widely used for the cytoplasmic expression of recombinant genes in mammalian cells. Vaccinia virus mutants and other poxviruses are receiving special attention because of their diminished cytopathic effects and increased safety. For example, replicating and non-replicating vectors encoding the bacteriophage T7 RNA polymerase for transcription of recombinant genes and numerous cancer antigens have been engineered (Carroll, M.W. and Moss B., *Curr. Opin. Biotechnol., 8(5)*:573-577 (1997); Carroll, M.W., *et al., Vaccine, 15(4)*:387-394 (1997).

The invention also relates to a method of monitoring the effectiveness of a vaccinia, variola and/or other related pox virus vaccine in an individual who has been administered the vaccinia vaccine. The method comprises determining whether the individual's T cells become activated in the presence of a polypeptide selected from the group consisting of: peptide 74A (SEQ ID NO: 1), peptide 165 (SEQ ID NO: 2), an immunogenic mutant or fragment thereof and a combination thereof, wherein if the individual's T cells become activated, then the virus is effective in the individual. In one embodiment, the vaccine is a vaccinia vaccine. In another embodiment, the vaccine is vaccinia virus that is a cancer vaccine.

EXEMPLIFICATION

Donors

Donors in this study were three HLA-A0201-positive laboratory workers received primary immunization by scarification with the licensed smallpox vaccine, Dryvax®, as recommended by the Centers for Disease Control and Prevention for laboratory personnel working with vaccinia viruses. The HLA-A and B alleles of donor 1 were A2 (A0201), B15, B18; those of donor 2 were A2 (A0201), B15, B44; and those of donor 3 were A2 (A0201), A31, B40, B51.

Viruses

Vaccinia virus New York City Board of Health (NYCBH), the same strain used to produce Dryvaxâ, was provided by Gail Mazzara and Dennis Panicali of Applied Biotechnology, Inc, and propagated and titrated in CV-1 cells (ATCC # CCL-70) as previously described (Littuau, R.A., *et al., J. Virol.*, 66:2274-2280 (1992); Terajima, M. *et al., Virus Res.* 84:67-77 (2002)). Modified vaccinia virus Ankara strain (MVA) was kindly supplied by Bernard Moss of National Institute of Allergy and Infectious Diseases/National Institute of Health, and was propagated and titrated in BHK-21 cells (ATCC # CCL-10) following published methods (Carroll, M.W. , *et al., Virology* 238:198-211 (1997)).

CTL lines

Vaccinia virus-specific CTL lines were isolated from peripheral blood mononuclear cells (PBMCs) of immunized donors by limiting dilution cloning (Demkowicz, W.E., *et al., J. Virol.* 67:1538-1544 (1993)). Vaccinia virus NYCBH strain was used to stimulate PBMCs for cloning and to infect target cells for cytotoxicity assays. Cytotoxicity assays were performed as previously described (Frey, S.E., *et al., J. Med.* 346:1275-1280 (2002)). Hmy C1R A2.1 cells (gift from William E. Biddison of NIH/NINDS), which express only HLA-A0201 at normal levels, were used as targets in cytotoxicity assay to confirm the HLA-A0201 restriction. Surface expression of CD4 and CD8 was determined by flow cytometry using FITC-conjugated antibodies (Becton Dickinson). Cross-reactivity of CTL lines was determined using autologous B-LCLs (B-lymphoblastoid cell lines), that were infected with MVA as target cells in cytotoxicity assays.

Screening peptides in cytotoxicity assay

Peptides were synthesized with a Symphony automated peptide synthesizer at the Protein Core Facility in the University of Massachusetts Medical School or purchased from Mimotopes Pty. Ltd. When predicted epitopes overlapped, we made them as a longer peptide fragment. For technical reasons some of these screening peptides were made as a 13mer instead of a 9mer. In screening cytotoxicity assays, mixtures of five peptides were used and the concentration of each peptide was 5mg/ml. When the peptide recognized was longer than 9 amino acids, truncated 9mer peptide epitopes were constructed and analyzed in cytotoxicity assays. All peptides recognized were tested in dose-response experiments (Figure 1).

Tetramer staining

Peptide/HLA-A0201 tetramers were made in the Tetramer Core Facility in the University of Massachusetts Medical School following the protocol published previously (Catalina, M.D., *et al., J. Immunol.*, 167:4450-4457 (2001)). Each lot of tetramer was titrated using CTL lines specific to the peptide mixed with autologous PBMCs at a 1 to 10 (or 20) ratio to determine the optimal concentration for staining.

Interferon (IFN) -g ELISPOT assay

IFN-g ELISPOT assays were performed as previously described (Ennis, F., *et al., J. Infect. Dis.* 185:1657-1659 (2002)). For stimulation, PBMCs were incubated with vaccinia virus NYCBH strain at an MOI of 1 or with peptide at a final concentration of 10 mg/ml for 16 hours.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 74A.

2. The method of Claim 1, wherein the polypeptide is selected from the group consisting of MVA189R, Copenhagen B22R, Copenhagen C16L, Bangladesh-1975 D2L, India-1967 D1L, Garcia-1966 B1L, Brighton Red V212 or Zaire-96-I-16 NIR.

3. The method of Claim 1, further comprising a second polypeptide comprising peptide 165.

4. The method of Claim 1, wherein the immune response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

5. A method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 165.

6. The method of Claim 5, wherein the polypeptide is selected from the group consisting of MVA018L, Copenhagen C7L, Tian Tan TC7L, Bangladesh-1975 D11L, India-1967 D8L, Garcia-1966 B14L, Brighton Red V028 or Zaire-96-I-16 D10L.

7. The method of Claim 5, further comprising a second polypeptide comprising peptide 74A

8. The method of Claim 5, wherein the immune response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

9. A method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 74A, immunogenic fragments or mutants thereof.

10. The method of Claim 9, wherein the polypeptide is selected from the group consisting of MVA189R, Copenhagen B22R, Copenhagen C16L, Bangladesh-1975 D2L, India-1967 D1L, Garcia-1966 B1L, Brighton Red V212 or Zaire-96-I-16 N1R.

11. The method of Claim 9, further comprising a second polypeptide comprising peptide 165, immunogenic fragments or mutants thereof.

12. The method of Claim 9, wherein the immune response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

13. The method of Claim 9, wherein 1 to about 4 amino acids can be substituted without essentially detracting from the immunological properties of peptide 74A.

14. A method for immunizing an individual against infection by vaccinia and/or variola virus, the method comprising inducing an immune response against a polypeptide comprising peptide 165, immunogenic fragments or mutants thereof.

15. The method of Claim 14, wherein the polypeptide is selected from the group consisting of MVA018L, Copenhagen C7L, Tian Tan TC7L, Bangladesh-1975 D11L, India-1967 D8L, Garcia-1966 B14L, Brighton Red V028 or Zaire-96-I-16 D10L.

16. The method of Claim 14, further comprising a second polypeptide comprising peptide 74A, immunogenic fragments or mutants thereof.

17. The method of Claim 14, wherein the immune response is induced by administering a product selected from the group consisting of a polypeptide, a naked nucleic acid molecule encoding the peptide or a nucleic acid molecule, encoding the peptide, in a suitable vector.

18. The method of Claim 14, wherein 1 to about 4 amino acids can be substituted without essentially detracting from the immunological properties of peptide 165.

19. A method of identifying the presence of vaccinia or variola virus in a sample comprising determining whether T cells present in the sample become activated in the presence of a polypeptide selected from the group consisting of: peptide 74A (SEQ ID NO: 1), peptide 165 (SEQ ID N O: 2), an immunogenic mutant or fragment thereof and a combination thereof, wherein if the T cells become activated, then vaccinia or variola virus is present in the sample.

20. The method of Claim 19 wherein whether the T cells present in the sample become activated is determined using an assay selected from the group consisting of: a cytokine assay, a flow cytometry assay and a limiting dilution assay.

21. The method of Claim 20 wherein the assay is an ELISPOT assay or a tetramer staining assay.

22. The method of Claim 19 wherein the sample is selected from the group consisting of: blood, lymph and tissue.

23. The method of Claim 22 wherein the sample is a peripheral blood mononuclear cell sample.

24. A method of determining whether an individual has been infected with vaccinia or variola virus comprising determining whether the individual's T cells become activated in the presence of polypeptide selected from the group consisting of: peptide 74A (SEQ ID NO: 1), peptide 165 (SEQ ID N O: 2), an immunogenic mutant or fragment thereof and a combination thereof, and wherein if the individual's T cells become activated in the presence of the peptide, then the individual has been infected with vaccinia or variola virus.

25. The method of Claim 24 wherein the individual's T cells are present in a sample, and the sample is selected from the group consisting of: blood, lymph and tissue.

26. The method of Claim 25 wherein the sample is a peripheral blood mononuclear cell sample.

27. The method of Claim24 wherein whether the whether the individual's T cells become activated is determined using an assay selected from the group

consisting of: a cytokine assay, a flow cytometry assay and a limiting dilution assay.

28. The method of Claim 27 wherein the assay is an ELISPOT assay or a tetramer staining assay.

29. A method of monitoring the effectiveness of a vaccinia vaccine in an individual who has been administered the vaccinia vaccine, comprising determining whether the individual's T cells become activated in the presence of a polypeptide selected from the group consisting of: peptide 74A (SEQ ID NO: 1), peptide 165 (SEQ ID N O: 2), an immunogenic mutant or fragment thereof and a combination thereof, wherein if the individual's T cells become activated, then the vaccinia virus is effective in the individual.

30. The method of Claim 29 wherein the individual's T cells of the individual are present in a sample, and the sample is selected from the group consisting of: blood, lymph and tissue.

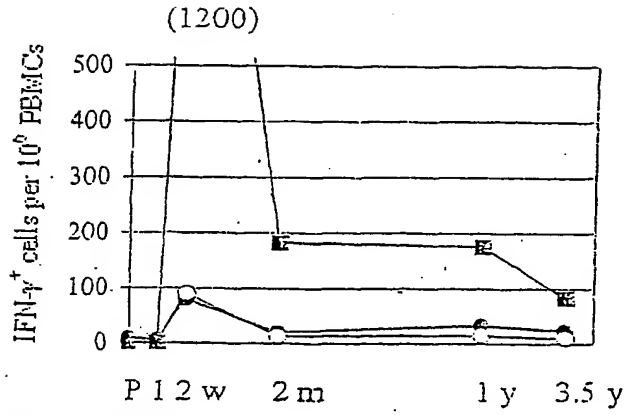
31. The method of Claim 30 wherein the sample is a peripheral blood mononuclear cell sample.

32. The method of Claim 29 wherein whether the whether the individual's T cells become activated is determined using an assay selected from the group consisting of: a cytokine assay, a flow cytometry assay and a limiting dilution assay.

33. The method of Claim 32 wherein whether the individual's T cells become activated is determined using an ELISPOT assay or a tetramer staining assay.

34. The method of Claim 29 wherein the vaccinia vaccine is a cancer vaccine.

Donor 1



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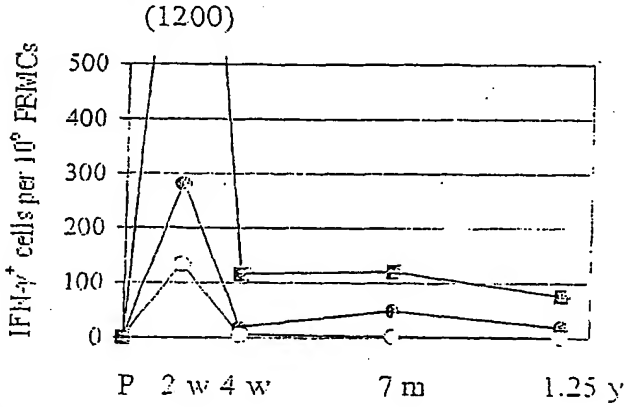

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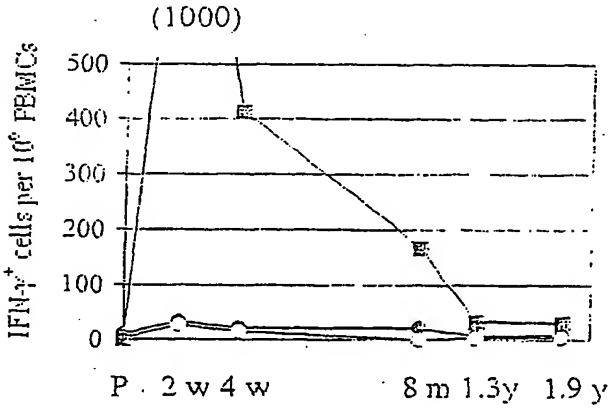

Fig. 1

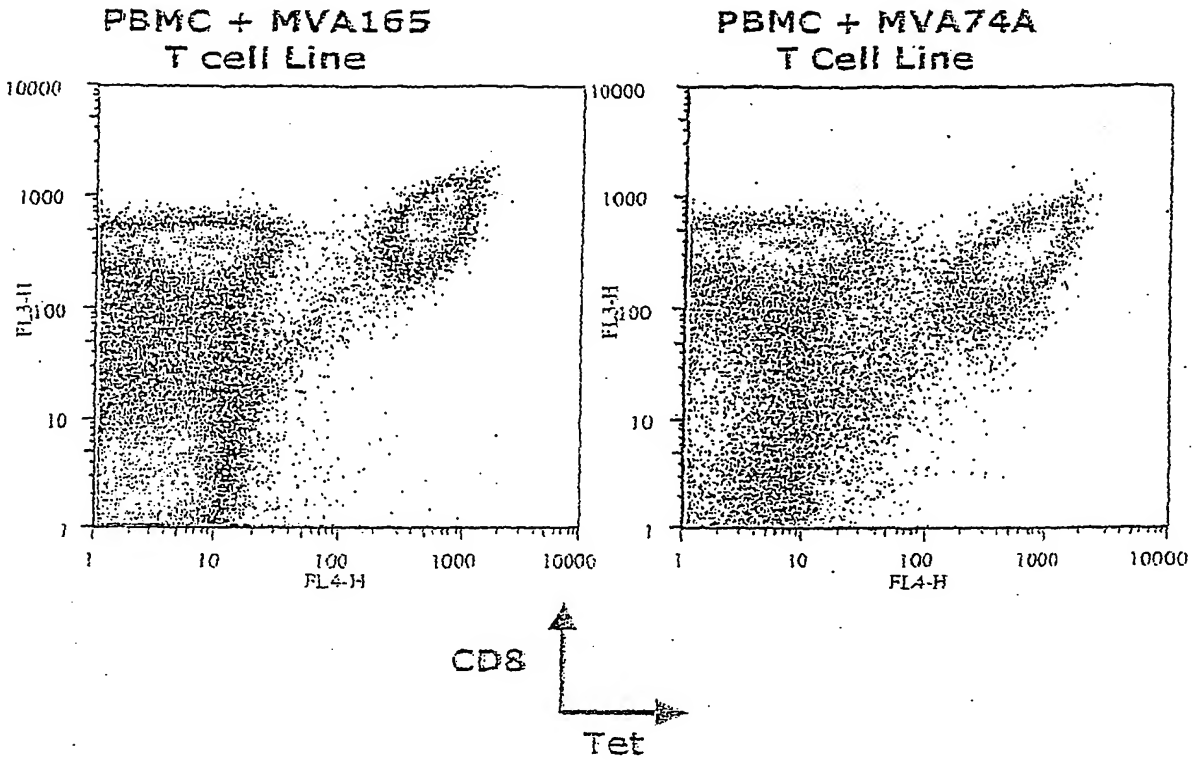

Fig. 2

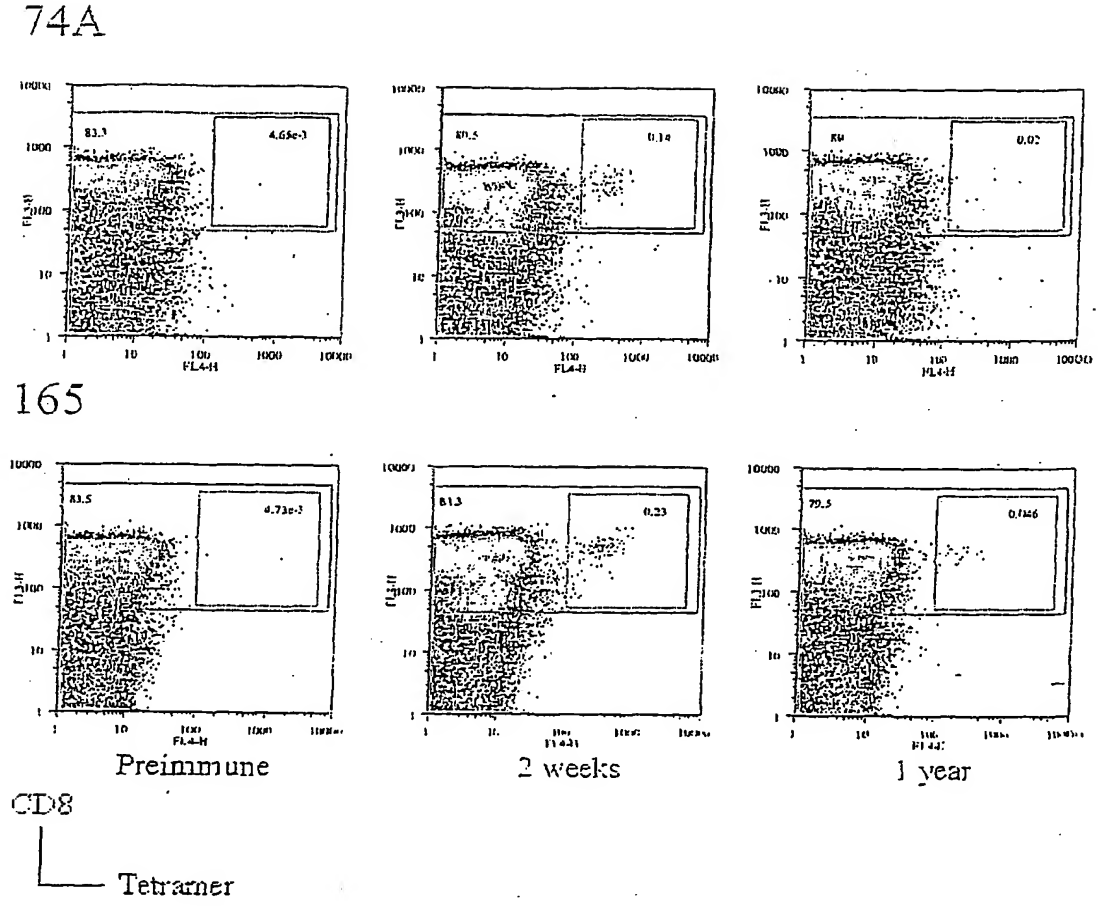

Fig. 3

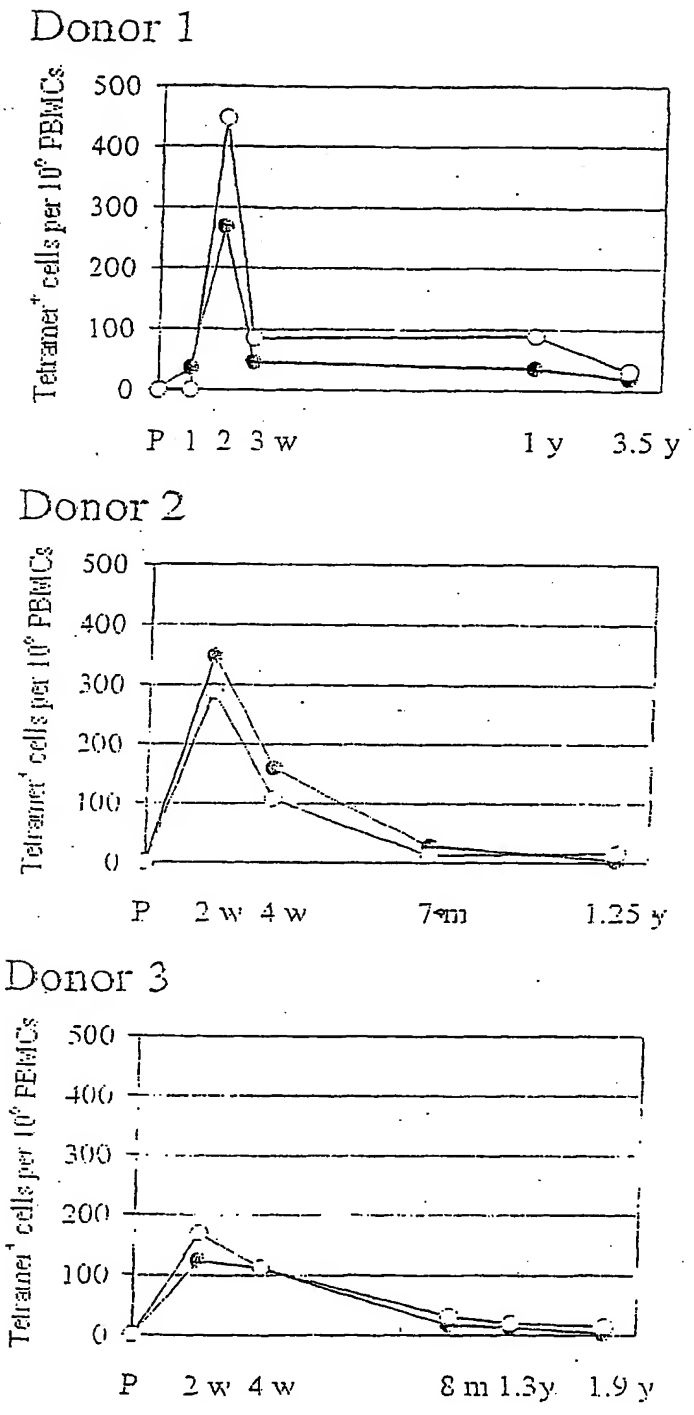

Fig. 4

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(54) Title: IDENTIFICATION OF GENE SEQUENCES AND PROTEINS INVOLVED IN VACCINIA VIRUS DOMINANT T CELL EPITOPES

(57) Abstract: The present invention relates to the identification of gene sequences and proteins involved in vaccinia virus dominant T cell epitopes. Two vaccinia virus $CD8^+$ T cell epitopes restricted by the most common human MHC class I allele, HLA-A0201 have been identified. Both epitopes are highly conserved in vaccinia and variola viruses. The induction of the T cell responses following primary vaccination is demonstrated by the kinetics of epitope specific $CD8^+$ T cells in 3 HLA-A0201 individuals. This information will be useful for the design and analyses of the immunogenicity of experimental vaccinia vaccines, and for basic studies of human T cell memory.

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INTERNATIONAL SEARCH REPORT

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/068682 A (SAHIN UGUR ; TUERECI OEZLEM (DE); LUDEWIG BURKHARD (DE)) 6 September 2002 (2002-09-06) C7L in Table 3; SeqIdNo.14; claims 26-29 --- -/--	5,6,8, 14,15, 17,19-33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
13 August 2004	14/09/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Lonnoy, O

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DREXLER I ET AL: "Identification of vaccinia virus epitope-specific HLA-A*0201-restricted T cells and comparative analysis of smallpox vaccines" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 100, no. 1, 7 January 2003 (2003-01-07), pages 217-222, XP002232973 ISSN: 0027-8424 page 218, column 2, paragraph 2	5,6,8, 14,15, 17,19-33
E	-& EP 1 398 380 A (GSF FORSCHUNGSZENTRUM UMWELT) 17 March 2004 (2004-03-17) SeqIdNo.48; claims 1,22	5,6,8, 14,15, 17,19-33
E	& WO 2004/024756 A (SUTTER GERD ; DREXLER INGO (DE); ERFLE VOLKER (DE); STAIB CAROLINE (DE) 25 March 2004 (2004-03-25) ---	5,6,8, 14,15, 17,19-33
P,X	TERAJIMA MASANORI ET AL: "Quantitation of CD8+ T cell responses to newly identified HLA-A*0201-restricted T cell epitopes conserved among vaccinia and variola (smallpox) viruses." THE JOURNAL OF EXPERIMENTAL MEDICINE. 7 APR 2003, vol. 197, no. 7, 7 April 2003 (2003-04-07), pages 927-932, XP002292276 ISSN: 0022-1007 the whole document ---	1-34
A	RAMIREZ JUAN C ET AL: "Biology of attenuated modified vaccinia virus Ankara recombinant vector in mice: Virus fate and activation of B- and T-cell immune responses in comparison with the Western Reserve strain and advantages as a vaccine" JOURNAL OF VIROLOGY, vol. 74, no. 2, January 2000 (2000-01), pages 923-933, XP002292277 ISSN: 0022-538X --- -/--	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMBL E.B.I. HINXTON U.K.; 2 November 1990 (1990-11-02) GOEBEL ET AL: "Vaccinia virus, complete genome." Database accession no. M35027 XP002292378 cited in the application complement (18805..19257): CL7; 181199..181744: B22R abstract	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2004/002141

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 1-18 are directed to methods of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
Although claims 24, 27, 28,
29 and 32-34, as far as these relate to methods practiced in vivo, are directed to diagnostic methods practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US2004/002141

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 02068682 A	06-09-2002	DE 10108626 A1	05-09-2002
		CA 2444493 A1	06-09-2002
		WO 02068682 A2	06-09-2002
		EP 1362125 A2	19-11-2003
		US 2004132132 A1	08-07-2004